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Genetics

The isolation of molecular genetic markers for the identification of sex

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ABSTRACT **It is difficult to identify sex in many species of eukaryotic organism. This can considerably impede research into their biology. Fortunately, one sex often possesses a unique chromosome termed Y or W. When DNA markers are available for these chromosomes, then sex identification becomes straightforward. We describe a technique that facilitates the isolation of such markers. The procedure makes use of low-stringency PCR to screen randomly selected primers for their ability to amplify sex-specific loci.**

A full understanding of the biology of any sexually reproducing species is dependent on being able to recognize male from female. This is because sex usually forms the largest single division within a species and causes differences in physiology, behavior, and ecology. At a more applied level, our ability to identify sex is pivotal in the design of breeding programs, whether for industrial or conservation purposes.

Unfortunately, the genetic sex of many species cannot be deduced from external morphology—a problem usually exacerbated when dealing with embryonic or juvenile forms, or when only small tissue samples are available.

One effective solution is to exploit DNA markers to diagnose sex. Such markers are present in the genome whenever sex determination is genetically controlled. In many organisms sexual differentiation is governed by chromosomal sex determination, where the sex-determinant genes are carried on a specialized pair of sex chromosomes. The two main forms are male heterogamety, where the male has X and Y chromosomes and the female is XX, and female heterogamety, where the female is WZ and the male is ZZ. The Y or W chromosomes are, thus, unique to one sex, so their presence or absence in a sample of genomic DNA is indicative of sex.

Sex identification at the DNA level is a well-established technique that has shown itself to be fast and accurate (1–3). It requires only minimal tissue samples (1) and these can be collected in the field and preserved under ambient conditions for long periods prior to analysis (3, 4). The only serious drawback is the difficulty of obtaining Y- or W-linked markers.

For mammals, this problem has largely been solved by the discovery of the *SRY* gene (*S*ex-determining *R*egion—*Y* chromosome) which is structurally conserved and Y-linked across the class (5, 6). For nonmammals, no widely conserved sex-specific genes have yet been described, and molecular sex identification in such organisms has been based on "junk" DNA sequences (7–9). These are perfectly adequate but are not usually conserved, so a sex-specific marker isolated from one species may not even exist outside the genus (8, 9). The technique described here makes the isolation of Y or W chromosome markers a relatively simple task and so should allow molecular sex identification to be more widely applied.

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MATERIALS AND METHODS

DNA was isolated from blood as described (8). PCR amplifications were carried out in 10 μl containing 20–100 ng of genomic DNA, each dNTP at 200 μM, 5–10 pmol of each primer, 0.35 units of *Taq* DNA polymerase, 50 mM KCl, 10 mM Tris·HCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100. The thermal profile for amplification of the great tit (*Parus major*) female-specific motif with the 23-mer primer ss2 (5′-CGGTCGGGAGGTTTCAAGGAATG-3′) was 95°C for 1.5 min; 38 cycles of 2 min at 47°C, 2 min at 72°C, and 1 min at 94°C; and final 5-min annealing and extensions steps at 47°C and 72°C, respectively. Two 10-mer primers (Operon) AB09 (GGGCGACTAC) and AB18 (CTGGCGTGTC) were used to amplify female-specific fragments from the jackdaw (*Corvus monedula*) and the zebra finch (*Taenopygia guttata*), respectively; the thermal profile for amplification was 95°C for 1.5 min; 38 cycles of 1 min at 37°C, 3 min at 72°C (with a ramp rate of 6 s/degree between annealing and extension steps), and 1 min at 94°C; and final 5-min annealing and extension steps at 37°C and 72°C, respectively. PCR products were visualized after electrophoresis through a 1.4% agarose gel. Female-specific amplified sequences were extracted from the agarose gels (10) to probe Southern blots. Blots were prepared from genomic DNA digested with either *Pvu* II or *Mbo* I (BRL), electrophoresed, and transferred to Zetaprobe GT membrane following instructions provided by the manufacturer (Bio-Rad). Hybridizations were carried out in aqueous buffer (11), and subsequent washes were performed at 65°C in 0.1% SDS/0.5× SSC [1× SSC is 0.15 M NaCl/15 mM sodium citrate (pH 7.5)].

RESULTS AND DISCUSSION

The procedure for identifying sex-specific loci requires two DNA samples, one derived from several males and the other from several females of the species. Each sample is subject to low-stringency PCR amplification with a single 10-mer primer or a mixture of two 20- to 30-mer primers of arbitrary sequence. This allows reproducible amplification of a range of DNA fragments that are amplified from the genome wherever opposed priming sites occur within 2–3 kb (12, 13). The two samples are then electrophoresed in adjacent lanes of an agarose gel where up to three types of product can be observed.

The first type of product results in bands of identical size in both male and female samples. These are derived from the amplification of an autosomal or Z/X-linked locus shared by the sexes. The second type is less common and gives a band only in the heterogametic sex. These are derived from W/Y-linked loci and form the sex-specific markers sought. A third type are those amplified from polymorphic loci that may yield a band in either one lane or the other. The use of pooled DNA samples is designed to minimize the effects of such individual variation.

This screening process is continued with different primers until one yields a type 2 sex-specific band. To confirm that this band is not the product of a rare polymorphism, PCR with this primer is then repeated with DNA samples from several sexed individuals.

The final stage of this process is illustrated for three species: the great tit, the jackdaw, and the zebra finch. To identify a female-specific marker in the great tit, it was necessary to screen a pool of 16 primers (21- to 29-mers) which had been designed for other purposes. Of these, primer ss2 produced, among others, a band of 724 bp (Fig. 1). For the jackdaw and zebra finch, 20 10-mer primers (of the Operon "AB" set) were screened to produce female-specific PCR products of 1100 bp (AB09) and 900 bp (AB18) in the two species, respectively (Fig. 1). For each species, the female-specific band occurred in each of five or more known females and was not present in five or more known males.

Each of the amplified, sex-linked fragments was then isolated and used to probe male and female genomic Southern blots. This confirmed that all were W-linked and gave some indication of their copy number and the occurrence of related sequences elsewhere in the genome (Fig. 2). In the great tit, the probe hybridized strongly to a W-linked fragment of 4.9 kb and less well to two larger fragments shared by both sexes. The probe isolated from the jackdaw hybridized to two W-derived sequences of 3.9 and 5.5 kb and rather more strongly to three other fragments present in both males and females. This suggests the latter occurred at a greater copy number than the W-linked target. The hybridization of the probe derived from the zebra finch was principally to autosomal or Z-linked sequences, suggesting that this motif is common in the zebra finch genome. Hybridization to a W-enriched fragment of 0.9 kb is, however, clearly apparent.

Once a primer has been shown to amplify a sex-specific sequence, there are a number of ways it can be used to identify sex. Three of the easiest options are:

(*i*) It can be used directly in low-stringency PCRs. This has the advantage that coamplified, non-sex-specific fragments act as positive controls. This ensures that the absence of a band is not interpreted as evidence for the sample coming from a homogametic individual when, in fact, the PCR has failed. This is particularly important in low-stringency PCR, which can have a higher failure rate than PCR with dedicated primers.

(*ii*) The fragment produced during the initial screening procedure can be sequenced and dedicated primers made. Nested primers allow amplification from the DNA of a few cells whilst maintaining specificity. Note that this strategy does require the separate design and use of non-sex-specific primers to act as a positive control.

(*iii*) If sample DNA is plentiful and Southern blot analysis has confirmed that the sex-linked DNA marker is unique to or highly enriched in the heterogametic sex, then hybridization to dot blots can be a rapid means of processing large numbers of samples (3).

A success rate for this method of isolating sex-specific markers is difficult to predict. It is dependent on the proportion and complexity of Y or W unique sequences in the genome. Also important is the length of the primers employed. As length decreases, primers theoretically should encounter a greater number of target sites and so increase the chance of amplifying a sex-specific locus. Given a sufficient number of primers, it should be possible to isolate a sex-specific marker from any organism where one sex possesses a unique chromosome.

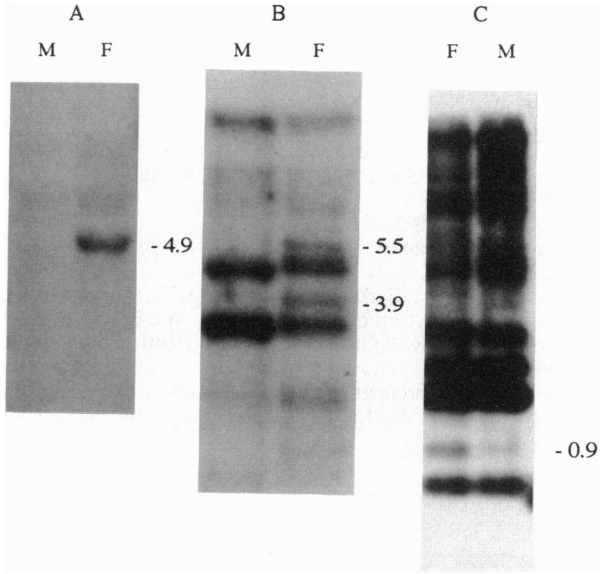

FIG. 2. Genomic Southern blots of DNA from individual male (lanes M) and female (lanes F) subjects. (*A*) DNA from great tits digested with *Pvu* II. (*B*) DNA from jackdaws digested with *Pvu* II. (*C*) DNA from zebra finches digested with *Mbo* I. The digestion products were probed with the female-specific PCR products produced for each species (see Fig. 1). All show hybridization consistent with a W chromosomal location of the target sequence (see text). In jackdaws and zebra finches, significant hybridization to sequence-related autosomal loci is also evident. Sizes are shown in kb.

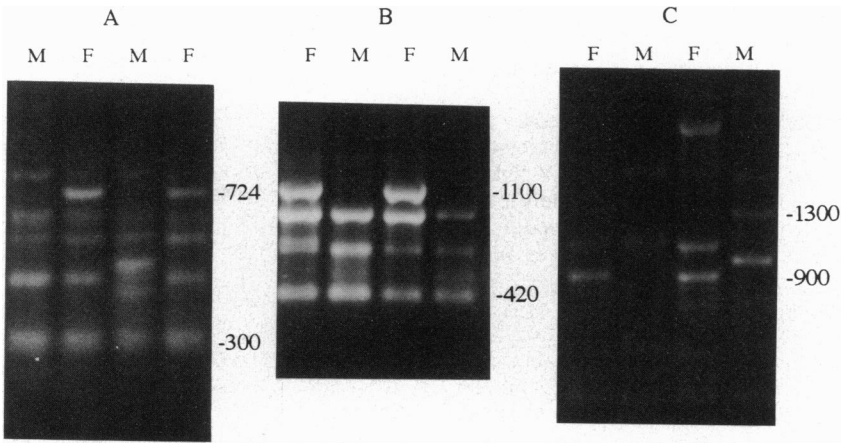

FIG. 1. (*A*) Low-stringency PCR amplification from the genomic DNA of two male (lanes M) and two female (lanes F) great tits with the primer ss2 produces a female-specific band of 724 bp. (*B* and *C*) Two 10-mer primers AB09 and AB18 produce female-specific fragments of ≈1100 bp in the jackdaw and ≈900 bp in the zebra finch, respectively. The 420-bp band in the jackdaws is an example of a single locus amplified in all individuals, while that of ≈1300 bp in the fourth jackdaw represents the amplification of a polymorphic locus.

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